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 Novel Genetic Models to Study the Role of Inflammation in Brain Injury-Induced Alzheimer's Pathology

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13. SUPPLEMENTARY NOTES

14. ABSTRACT
Individuals exposed to traumatic brain injury (TBI) are at a greatly increased risk for developing a number of neurodegenerative diseases including Alzheimer's disease (AD). TBI promotes the development of the pathological hallmarks of AD including production and extracellular deposition of the beta -amyloid peptide in senile plaques and intracellular aggregation of hyperphosphorylated, microtubule-associated protein tau (MAPT) in neurofibrillary tangles (NFTs). Several lines of evidence suggest that altered monocyte infiltration and microglial activation may be directly involved in the pathogenesis of both beta-amyloid and MAPT pathologies. The primary hypothesis to be tested in the current studies is that TBI induces infiltration of peripheral monocytes as well as acute and local activation of brain microglia within the injured brain and that these two cell types play roles distinct from each other in inducing both beta-amyloid pathologies and MAPT phosphorylation and aggregation leading to chronic pathological conditions that pre-dispose individuals exposed to TBI to develop AD later in life. Here we report that TBI results in brain cavitation and a widespread neuroinflammatory response including microglial activation and monocyte infiltration. Interestingly, the acute macrophage response to TBI is reduced in a mouse model of amyloid pathology (R1.40) compared to control mice; however, long-term behavioral outcome and neurodegeneration are worse at chronic postinjury time points. By contrast, pilot data examining TBI in hTau mice revealed increased phosphorylation of tau within neurons as well as enhanced microglial activation including swollen cell bodies and thickened processes when compared to brain injured controls. Recent data suggests that the macrophage response is also enhanced in brain injured hTau mice at acute postinjury time points and behavioral assessment reveals deficits in spatial references memory at chronic time points. Taken together, these novel results suggest that TBI impacts amyloid and MAPT pathologies quite differently and have implications for immunomodulatory therapies for TBI.

15. SUBJECT TERMS
Alzheimer's, brain injury, beta-amyloid, MAPT, inflammation, monocytes, microglia

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Introduction
Exposure to traumatic brain injury (TBI) has numerous acute and chronic sequelae, including an increased risk for the development of Alzheimer's disease (AD)[1-3]. Mechanisms contributing to these pathological events are not well characterized; however, they are often associated with neuroinflammation. Postinjury neuroinflammation is characterized by activation of brain-resident microglia as well as infiltration and activation of monocytes due to disruption of the blood-brain barrier [4-6]. Assessing the exact roles of these cells in AD pathogenesis has proven exceedingly difficult as no immunohistochemical markers unambiguously differentiate monocytes from microglia, particularly in the activated (macrophage) state. Although there is increasing experimental evidence that macrophages derived from recruited monocytes can regulate Aβ pathologies, the effects of monocytes on other AD phenotypes, or the role of these cells in TBI induced AD pathologies have yet to be established. The central hypothesis of the current studies is that monocytes and microglia play roles decisively distinct from each other in the development of AD-like pathologies following TBI. Furthermore, these functions are exerted differentially in the varied pathological processes that underlie AD, including generation and extracellular deposition of beta-amyloid (Aβ) and phosphorylation and intracellular aggregation of microtubule associated protein tau (MAPT).

Body
Upon receipt of this award, we prepared and submitted a protocol to the Cleveland Clinic Institutional Animal Care and Use Committee (IACUC), which contained a detailed description of all studies proposed in the grant. The protocol was approved after minor revisions. The same animal use protocol was reviewed by the USMRMC Office of Research Protections. After additional minor revisions, including clarification of multiple survival surgeries and animal monitoring, all procedures were approved. It should be noted that we recently submitted a request for a one-year no-cost extension to account for unforeseen delays in hTau breeding and optimization of a new flow cytometry protocol. Importantly, we are now well-position to complete the proposed studies during the extension.

Specific Aim 1: Examine the spatial and temporal appearance of neuroinflammation and β-amyloid (Aβ) pathologies, cognitive deficits and neurodegeneration in R1.40 mice subjected to the lateral fluid percussion injury (FPI) paradigm of TBI

Task 1. Generate animals required for studies; Completed Quarter 30-09-2012 to 31-12-2012
Task 2. Perform FPI; Completed Quarter 01-01-2013 to 31-03-2013
Task 3. Perform behavioral analysis; Completed Quarter 01-04-2013 to 30-06-2013
Task 4. Analysis of brain tissue; Completed Quarter 01-04-2013 to 30-06-2013
Task 5. Published manuscript on results from Specific Aim 1; Initiated Quarter 01-10-2013 to 31-12-2013 (revisions are being made for resubmission)

The Lamb lab has generated the R1.40 mouse model of AD, which contains a full genomic copy of the human amyloid precursor protein (*APP*) gene with a familial AD mutation and develops age-related appearance of Aβ pathology at 12 months of age as well as earlier biochemical, neuropathological, and behavioral features of human AD [7, 8]. The R1.40 model is used extensively in the Lamb lab and therefore we were able to easily generate the animals required for the proposed studies. All R1.40 and non-transgenic animals (C57BL/6J, [B6]) generated were aged to two-months old before being surgically prepared for sham injury or lateral FPI resulting in four injury groups: R1.40 Sham, R1.40 TBI, B6 Sham, B6 TBI. Half of the animals in each group were aged to the acute 3 days postinjury (DPI) time point and the other half were aged to the chronic 120 DPI time point. Animals aged to 120 DPI completed motor and cognitive tests prior to sacrifice. At the selected time points, all mice were transcardially perfused and brain tissue was subsequently processed for neuropathological or biochemical analysis to document changes in postinjury neuroinflammation and AD-like Aβ pathology.

All of the experiments for **Specific Aim 1** were completed during the first year of the grant. Major findings were described in the first annual report. A manuscript describing these studies was prepared and submitted for publication. Subsequent additional experiments were performed to address various comments from reviewers. These data were included in the last quarterly report and ultimately compliment the initial findings, indicating that the presence of accumulating Aβ alters the post-injury macrophage response in R1.40 mice compared to Non-Tg mice. Our data confirmed that this response was independent of tau pathology as well as astrogliosis. Multiplex cytokine array revealed genotype differences in pro-inflammatory cytokines; however,

few significant differences between TBI and sham groups were detected. Final revisions are being made and the manuscript will be resubmitted to *Science Translational Medicine* by November 1, 2014.

Specific Aim 2: Examine the spatial and temporal appearance of neuroinflammation and MAPT pathologies, cognitive deficits and neurodegeneration in hTau mice subjected to lateral FPI.

Task 1. Generate animals required for studies; Completed Quarter 01-01-2013 to 31-03-2013 (following generation of these mice, breeding declined and delayed subsequent studies)
Task 2. Perform FPI; Initiated Quarter 01-10-2013 to 31-12-2013 and Completed Quarter 01-01-2014 to 31-03-2014
Task 3. Perform behavioral analysis; Initiated 01-01-2014 to 31-03-2014; Completed Quarter 01-10-14 to 31-12-14
Task 4. Analysis of brain tissue; Initiated 01-01-2014 to 31-03-2014; Expected Completion months 24-27
Task 5. Published manuscript on results from Specific Aim 2; Expected Completion months 27-30

Davies and colleagues developed a genomic mouse model of human MAPT pathology (termed hTau) by expressing the entire wild-type human *MAPT* gene in mice lacking the endogenous *Mapt* gene [9, 10]. hTau mice exhibit age-related accumulation of hyperphosphorylated human MAPT as well as altered neuronal cell cycle and cell death with appropriate localization, timing and neurotoxic effects. This unique model allows us to accurately study the effects of TBI induced inflammation on disease-relevant human MAPT pathology. The hTau model is used extensively in the Lamb lab; however, there were some unforeseen difficulties in breeding. As a result our initial efforts were focused on utilizing the hTau and matched control mice to assess neuropathoogical changes at the acute 3 DPI time point. Fortunately, breeding substantially improved and we have been able to complete all studies for **Specific Aim 2**.

We also experienced a slight delay in late 2013, when we switched from using a fluid percussion injury (FPI) device manufactured by Dragonfly (http://www.dragonflyinc.com/fluid_perc.htm) in **Specific Aim 1** to one manufactured by Amscien (http://amscien.com/AmsFluid.htm) in **Specific Aims 2 and 3**. Sudden inconsistencies in the Dragonfly FPI device resulted in variable brain injuries and increased mortality. The Amscien FPI device is located in Dr. Imad Najm's laboratory at the Cleveland Clinic and proved to be a valuable tool in completion of the remaining studies. We maintain a collaborative working relationship with the Najm laboratory and will continue to use the Amscien device for all remaining studies. Ultimately, we feel this change has improved the overall quality of our experiments. We received some criticism for creating a severe instead of a moderate TBI with the Dragonfly device, as evidenced by the large injury cavity at 3 DPI following a 1 ATM TBI. A representative image from an hTau mouse reveals that a 1 ATM TBI with the Amscien FPI causes less tissue damage, which is more clinically relevant (**Fig. 1**). A caveat to this change is that we cannot compare results in the hTau mice to those in the R1.40 mice because the injury severity is clearly different. However, R1.40 mice are readily available in the Lamb lab and several have been injured with the Amscien device to ensure post-injury outcome is consistent.

Neuropathological and biochemical analysis of brain tissue from Non-Tg and hTau mice at 3 and 120 DPI is ongoing and will be completed over the next several months (as indicated by our revised **Statement of Work**). Initial immunohistochemical data indicates that, as expected, the macrophage response is enhanced in hTau TBI mice compared to Non-Tg TBI mice (**Fig. 2**); however, several more animals need to be added to the analysis before final conclusions can be made. The final cohort of mice completing behavioral testing for the chronic 120 DPI time point were sacrificed in October, 2014. Most behavioral data has been analyzed at this time. No significant differences were detected between groups in rotarod (**Fig. 3)** or y maze performance (**Fig. 4**) at any post-injury time point. hTau TBI mice took significantly longer than hTau sham mice to reach the hidden platform in the water maze task, but no other significant differences in latency to the platform were detected between groups (**Fig. 5**). Distance and probe trial data still need to be analyzed before final conclusions can be made. At this time, separate graphs of Non-Tg and hTau mice are shown. This was done to more clearly display the data. Graphs with all four experimental groups are crowded and unique group differences are obstructed. Animals from all four experimental groups (N = 12-14 mice/group) were run through behavioral testing at the same time and statistical analysis included animals from all four groups.

Specific Aim 3: Identify, track, purify and analyze gene expression profiles on infiltrating monocytes and resident microglia at different stages of Aβ and MAPT pathologies following TBI.

Statement of Work:
Task 1. Generate animals required for studies; Completed Quarter 01-07-13 to 30-09-2013
Task 2. Perform FPI; Completed Quarter 01-01-2014 to 31-03-2014
Task 3. Analysis of brain tissue; Initiated Quarter 01-01-2014 to 31-03-2014; Expected Completion months 24-27
Task 4. Purification of monocytes/microglia; Initiated Quarter 01-01-2014 to 31-03-2014; Expected Completion months 24-27
Task 5. Gene expression microarray analysis; Expected Completion months 27-30
Task 6. Multi-photon microscopy; Expected Completion months 24-27
Task 7. Published manuscript on results from Specific Aim 1; Expected Completion months 27-30

In blood, two non-overlapping monocyte populations are distinguished by differential expression of two chemokine receptors and surface glycoprotein Ly6C (Ly6C^{hi}/CX3CR1^{low}/CCR2+ and Ly6C^{low}/CX3CR1^{hi}/CCR2-negative)[11, 12]. No tissue-immunohistochemistry markers unambiguously differentiate monocytes from microglia, particularly in the activated (macrophage) state [13]. However, our laboratories have recently performed pioneering studies using genetic labels regulated by these chemokine-receptor promoters to show that, serendipitously, CX3CR1 and CCR2 distinguish microglia from monocytes in the inflamed CNS [14]. This distinction arose from the following observations:

- Monocyte expression of CCR2, responding the chemokine ligand CCL2, is functionally required for monocytes to enter the CNS and therefore CNS-infiltrating monocytes are CCR2+;
- CX3CR1 is expressed by microglia from their first entry into the neuroepithelial parenchyma around E10 until adulthood, when microglia remain uniformly CX3CR1+. Furthermore, microglia do not express CCR2 or downregulate CX3CR1, even during severe neuroinflammation.

Cx3cr1$^{GFP/+}$/Ccr2$^{RFP/+}$ mice provided a first opportunity to evaluate differential microglial and monocyte contributions to acute neuroinflammation by correlating gene expression of sorted monocytes and microglia with protein localization by immunohistochemistry (IHC). Cx3cr1$^{GFP/+}$/Ccr2$^{RFP/+}$ mice were readily available within the Lamb and Ransohoff labs; however, R1.40/ Cx3cr1$^{GFP/+}$/Ccr2$^{RFP/+}$ and hTau/ Cx3cr1$^{GFP/+}$/Ccr2$^{RFP/+}$ were not. All needed genotypes have been created; however, we experienced significant delays in breeding hTau/ Cx3cr1$^{GFP/+}$/Ccr2$^{RFP/+}$ mice. All hTau/ Cx3cr1$^{GFP/+}$/Ccr2$^{RFP/+}$ mice thus far have been utilized for acute 3 DPI neuropathological analysis and we currently have N = 6-8. Future hTau/ Cx3cr1$^{GFP/+}$/Ccr2$^{RFP/+}$ mice will be given a TBI and aged to 120 DPI for neuropathological analysis. We also have several additional hTau TBI and sham mice aging to 120 DPI for flow cytometry studies. It is possible to sort microglia and monocytes without fluorescent tags if necessary and thus we feel confident that all proposed studies can be completed during the extension period. On the other hand, we have a stock supply of age matched Cx3cr1$^{GFP/+}$/Ccr2$^{RFP/+}$ and R1.40/ Cx3cr1$^{GFP/+}$/Ccr2$^{RFP/+}$ mice in our colony. Brain tissue for neuropathological analysis has been collected from TBI and sham Cx3cr1$^{GFP/+}$/Ccr2$^{RFP/+}$ and R1.40/ Cx3cr1$^{GFP/+}$/Ccr2$^{RFP/+}$ mice at 3 and 120 DPI. Final analysis is being completed at this time; however, the ipsilateral thalamus appears to be a particularly vulnerable subcortical brain structure to long-term damage following TBI as several RFP+ cells are detected at 120 DPI.

We have spent significant effort optimizing a new cell isolation protocol for flow cytometry studies in Cx3cr1$^{GFP/+}$/Ccr2$^{RFP/+}$ and R1.40/ Cx3cr1$^{GFP/+}$/Ccr2$^{RFP/+}$ mice. Use of the Amscien FPI device results in a more moderate TBI and ultimately decreases the number of infiltrating RFP+ cells. We considered pooling mice for gene expression studies and may still have to do that; however, optimization of a new cell isolation protocol has increased the number of isolated GFP+ and RFP+ cells as well as the integrity of these cells. We are currently working with the Genomics Core at the Cleveland Clinic to optimize RNA quality and foresee no difficulty in completing the gene expression studies in post-injury microglia and monocyte populations.

Finally, we have initiated multi-photon studies and have been working with the Imaging Core and other laboratories at the Cleveland Clinic to optimize post-injury imaging of GFP+ microglia and RFP+ monocytes. We have imaged at several post-injury time points and capture both GFP+ and RFP+ cells near the injury cavity. We will continue to work on these experiments and have even considered comparing these results with slice culture imaging to detect GFP+ and RFP+ cells in subcortical brain structures such as the hippocampus.

Key Research Accomplishments

- ***Approvals and Training of Staff***
  a. Task 1. Complete required approval documents for the studies; Completed Quarter 30-09-2012 to 31-12-2012
  b. Task 2. Provide required training for staff; Completed Quarter 30-09-2012 to 31-12-2012
- ***Specific Aim 1, Data analysis completed and awaiting manuscript review***
  a. Task 1. Generate animals required for studies; Completed Quarter 30-09-2012 to 31-12-2012
  b. Task 2. Perform FPI; Completed Quarter 01-01-2013 to 31-03-2013
  c. Task 3. Perform behavioral analysis; Completed Quarter 01-04-2013 to 30-06-2013
  d. Task 4. Analysis of brain tissue; Completed Quarter 01-04-2013 to 30-06-2013
  e. Task 5. Published manuscript on results from Specific Aim 1; Initiated Quarter 01-10-2013 to 31-12-2013 (revisions are being made for resubmission)
- ***Specific Aim 2, Tissue collection and behavioral analysis completed***
  a. Task 1. Generate animals required for studies; Completed Quarter 01-01-2013 to 31-03-2013 (following generation of these mice, breeding declined and delayed subsequent studies)
  b. Task 2. Perform FPI; Initiated Quarter 01-10-2013 to 31-12-2013 and Completed Quarter 01-01-2014 to 31-03-2014
  c. Task 3. Perform behavioral analysis; Initiated 01-01-2014 to 31-03-2014; Completed Quarter 01-10-14 to 31-12-14
  d. Task 4. Analysis of brain tissue; Initiated 01-01-2014 to 31-03-2014; Expected Completion months 24-27
  e. Task 5. Published manuscript on results from Specific Aim 2; Expected Completion months 27-30
- ***Specific Aim 3, all animals have been generated and a new optimized flow cytometry protocol will streamline gene expression studies***
  a. Task 1. Generate animals required for studies; Completed Quarter 01-07-13 to 30-09-2013
  b. Task 2. Perform FPI; Completed Quarter 01-01-2014 to 31-03-2014
  c. Task 3. Analysis of brain tissue; Initiated Quarter 01-01-2014 to 31-03-2014; Expected Completion months 24-27
  d. Task 4. Purification of monocytes/microglia; Initiated Quarter 01-01-2014 to 31-03-2014; Expected Completion months 24-27
  e. Task 5. Gene expression microarray analysis; Expected Completion months 27-30
  f. Task 6. Multi-photon microscopy; Expected Completion months 24-27
  g. Task 7. Published manuscript on results from Specific Aim 1; Expected Completion months 27-30

Reportable Outcomes

Abstracts

- Kokiko-Cochran, O., Veenstra, M., Ransohoff, L., Bhaskar, K., Lee, Y-S, Lamb, B. "Traumatic brain injury distinctly influences amyloid and tau pathology", Society for Neuroscience Conference, New Orleans, LA, October, 2012.
- Kokiko-Cochran, O., Ransohoff, L., Bhaskar, K., Lee, Y-S, Lamb, B. "Traumatic brain injury induces a distinct inflammatory response in a genomic based model of Alzheimer's disease", National Neurotrauma Symposium, Nashville, TN, August, 2013.
- Kokiko-Cochran, O., Saber, M., Teknipp, R., Ransohoff, R., Lamb, B. "Traumatic brain injury induces a distinct macrophage response at acute and chronic time points in a mouse model of Alzheimer's disease", selected for nanosymposium, Society for Neuroscience Conference, Washington, D.C., November, 2014.

Publications

- Manuscript describing experiments completed in Specific Aim 1 prepared "Altered neurodegeneration, neuroinflammation, and behavior following traumatic brain injury in a mouse model of Alzheimer's disease" to be submitted in the next week.

Animal Models
- CX3CR1$^{GFP/+}$/CCR2$^{RFP/+}$, R1.40/ CX3CR1$^{GFP/+}$/CCR2$^{RFP/+}$, and hTau/ CX3CR1$^{GFP/+}$/CCR2$^{RFP/+}$ mice generated and available to requesting investigators. All models are also deposited at The Jackson Laboratory.

Grant Funding
- NINDS R21, NS087298, PIs: B.T. Lamb and R.M. Ransohoff, "The Role of Monocytes and Microglia in Traumatic Brain Injury-Induced Tauopathies, 7/1/13-6/30/15.
- TATRC MRPRA, ERMS #13321017, PIs: L. Goldstein and B.T. Lamb, "Effects of Blast Neurotrauma on Alzheimer's Disease Pathogenesis, 10/1/13-9/31/15.
- TATRC MRPRA, ERMS #13267031, PI: B.T. Lamb, "The Role of TREM2 in Traumatic Brain Injury Induced Tauopathy"

Conclusion

Numerous studies have documented that prior incidence of TBI may gradually lead to AD-related pathologies, neuroinflammation, neurodegeneration and dementia. Since neuroinflammation is a rapid response to brain trauma, it is extremely important to understand specific contributions of microglia (resident immune cells of the brain) *versus* monocytes (bone-marrow derived immune cells) in mediating amyloid and MAPT pathology as well as cognitive impairment, which is often encountered in injured combatants. The first two specific aims include experiments to characterize the neuroinflammatory response in separate amyloid and tau mouse models of AD. Our data continues to suggest that the brain injury induced neuroinflammatory response impacts Aβ and MAPT pathologies quite differently, with only modest effects focused on altered microglial activation in a genomic-based model of Aβ pathologies, but dramatically enhanced MAPT phosphorylation and aggregation in a genomic-based model of MAPT pathologies, although proper quantification still needs to be completed. The final studies in this project will utilize and characterize unique genetic and genomic models to individually label microglia and monocytes so as to understand their relative contribution and functional roles in neurodegenerative processes. The outcome of this study will have a major impact in understanding the role of monocytes and microglia in AD-related pathologies following TBI. We will identify when these cells first appear following TBI, the genes expressed in each cell type and the spatial and temporal relationship to Aβ and MAPT pathologies. We currently have all genotypes necessary to finish this project and are well positioned to provide all deliverables listed in our Statement of Work included in the no-cost extension.

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Supporting Data

Dragonfly

Amscien

Non-Tg TBI   R1.40 TBI   Non-Tg Sham   hTau TBI
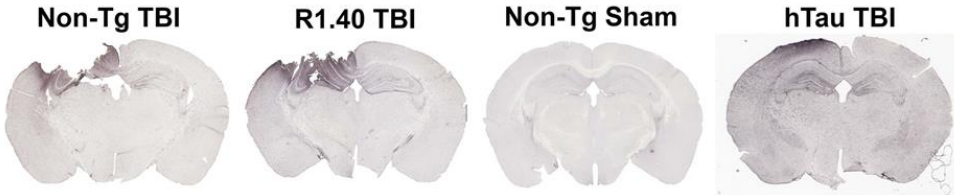
Figure 1. The Dragonfly FPI was used for all studies in **Specific Aim 1**. Representative images from Non-Tg TBI and R1.40 TBI mice are shown following Dragonfly FPI. A Non-Tg Sham mouse is shown to demonstrate minimal tissue damage following sham surgery. The Amscien FPI was adopted in late 2013, to complete studies in **Specific Aims 2 and 3**. A representative image from a brain injured hTau mice shown on the bottom bar right. Tissue damage is clearly decreased following TBI with the Amscien FPI. All TBI's, regardless of injury device, were created with 1 ATM of pressure.
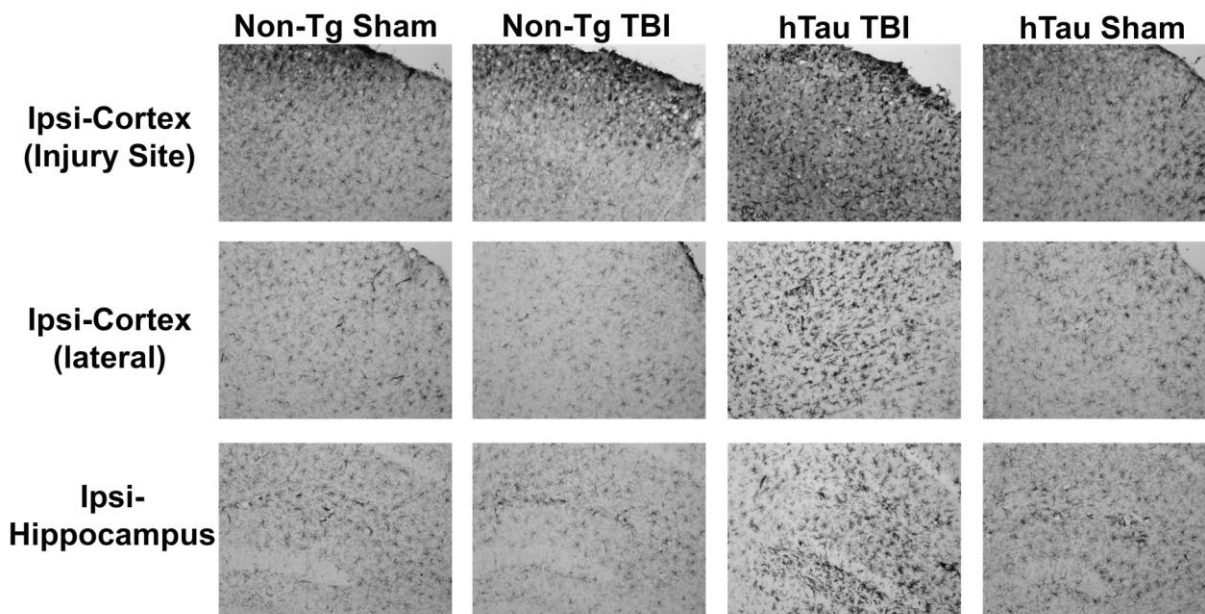

Figure 2. Non-Tg and hTau mice received a lateral FPI or sham injury at 2 months of age and were sacrificed at 3 DPI. Initial characterization reveals enhanced CD45+ staining around the site of injury and in the ipsilateral hippocampus of hTau TBI mice compared to Non-Tg TBI mice.
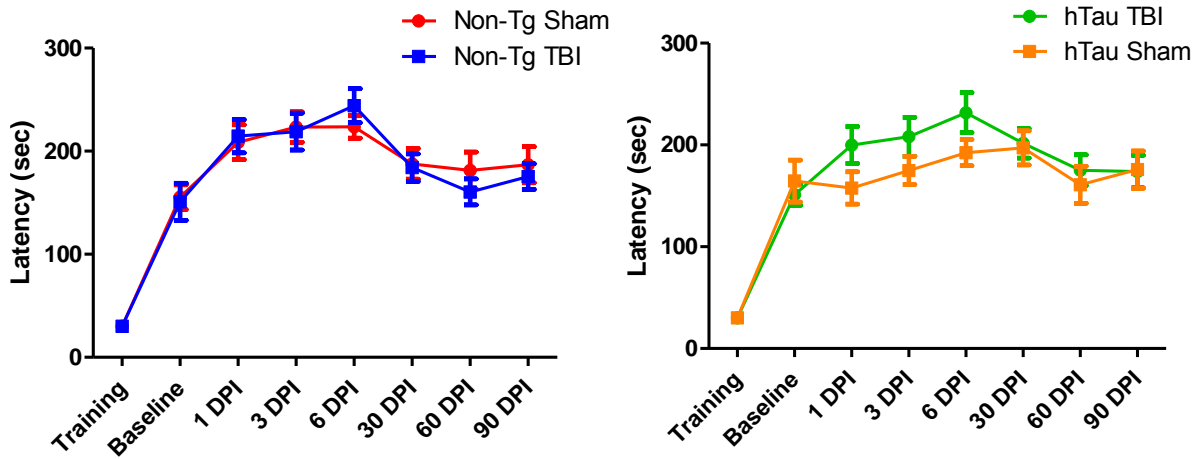
Figure 3. Post-injury balance and coordination was assessed with the rotarod at 1, 3, 5, 30, 60, and 90 DPI. No significant differences in latency to remain on the rotating rod were detected between groups across time points. Together these data demonstrate that this model of experimental TBI does not results in long-term post-injury motor deficits.
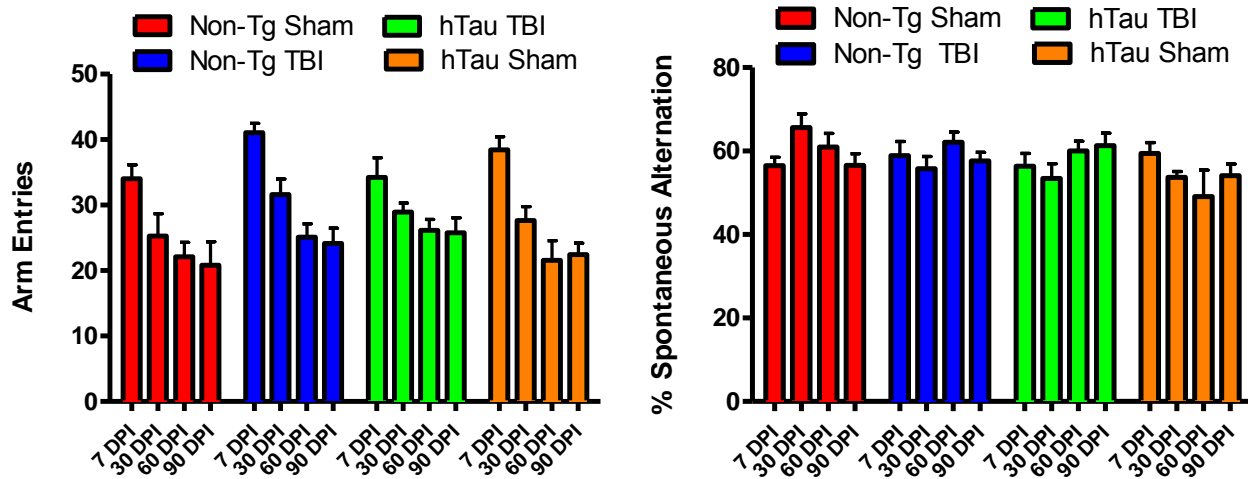

Figure 4. Post-injury spatial working memory was assessed with the y maze at 7, 30, 60, and 90 DPI. No significant differences in total arm entries were detected between groups at any time point; however, all groups demonstrated a reduction in arm entries following repeated exposure to the y maze over time. Furthermore, no significant differences between groups were detected in percentage of spontaneous alternations between arms. Together these results demonstrate that TBI does not result in spatial working memory deficits in Non-Tg or hTau mice.
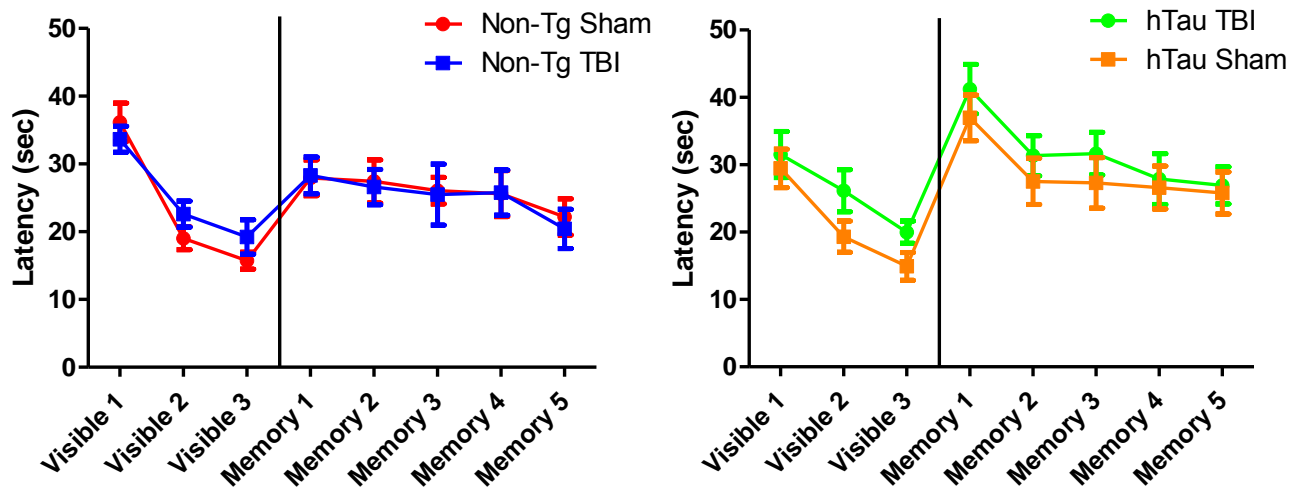
Figure 5. Post-injury spatial reference memory was assessed with the water maze task at 120 DPI. Animals received 3 days of visible platform training following by 5 days of memory testing. Non-Tg TBI and sham mice performed similarly during water maze testing; however, a significant difference in latency to reach the submerged goal platform was detected during memory testing in the hTau mice. These graphs clearly show that hTau mice, regardless of injury group, show a dramatic increase in latency to reach the platform on day 1 of memory testing compared to Non-Tg mice. Additional analysis is needed to identify specific aspects of cognitive function responsible for this behavior.